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IMMUNIZATION BY INOCULATION OF DNA TRANSCRIPTIONAL UNIT

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Background of the Invention

Vaccination with inactivated or attenuated organisms or their products has been shown to be an effective method for increasing host resistance and ultimately has led to the eradication of certain common and serious infectious diseases. The use of vaccines is based on the stimulation of specific immune response within a host or the transfer of preformed antibodies. The prevention of certain diseases, such as poliomyelitis, by vaccines represents one of immunologies greatest triumphs.

Effective vaccines have been developed for relatively few of the infectious agents that cause disease in domestic animals and man. This reflects technical problems associated with the growth and attenuation of virulent strains of pathogens. Recently effort has been placed on the development of subunit vaccines (vaccines that present only selected antigens from a pathogen to the host). Subunit vaccines have the potential for achieving high levels of protection in the virtual absence of side effects. Subunit

vaccines also offer the opportunity for the development of vaccines that are stable, easy to administer, and sufficiently cost-effective for widespread distribution.

Summary of the Invention

This invention relates to a method of immunizing an individual, comprising introducing into the individual a DNA transcriptional unit which comprises DNA encoding a desired antigen or antigens. The uptake of the DNA transcriptional unit by host cells results in the expression of the desired antigen or antigens thereby eliciting humoral or cell-mediated immune responses or both humoral and cell-mediated responses. The elicited humoral and cell-mediated immune response can provide protection against infection by pathogenic agents, provide an anti-tumor response, or provide contraception. The host can be any vertebrate, avian or mammal, including humans.

The DNA transcriptional unit introduced by the present method can be used to express any antigen encoded by an infectious agent, such as a virus, a bacterium, a fungus, or a parasite as well as "designer" antigens that have been experimentally determined to be able to be used to immunize against infection by a pathogenic agent. As stated above, DNA transcriptional units can also be used for contraceptive purposes or for anti-cancer therapy.

The desired antigen to be expressed can be designed so as to give internal, surface, secreted, or budding and assembled forms of the antigens being used

as immunogens.

There are numerous advantages for the use of DNA for immunizations. For example, immunization can be accomplished for any antigen encoded by DNA. Furthermore, the DNA encoded antigens are expressed as "pure" antigens in their native states and have undergone normal host cell modifications. Also, DNA is easily and inexpensively manipulated and is stable as a dry product or in solution over a wide range of temperatures. Thus, this technology is valuable for the development of highly effective subunit vaccines.

Brief Description of the Drawings

Figure 1 is an illustration of a bacterial plasmid containing a DNA transcriptional unit (referred to as pP1/H7) consisting of the influenza virus hemagglutinin type 7 (H7) gene expressed by a replication competent retroviral vector.

Figure 2 is an illustration of a bacterial plasmid containing a DNA transcriptional unit (p188) consisting of the influenza virus hemagglutinin type 7 (H7) gene expressed by a replication defective retroviral vector.

Figure 3 is an illustration of a bacterial plasmid containing the retroviral vector (pRCAS) with no H7 insert, used as a control.

Detailed Description of the Invention

This invention relates to a method of immunizing an individual against a pathogen, resulting in protection against subsequent challenge by the pathogen because the infectious agent is cleared by humoral and cell-mediated immune responses, thus limiting the spread or growth of the infectious agent.

The term "immunizing" refers herein as the production of an immune response in an individual which protects (partially or totally) from the manifestations of infection (i.e., disease) caused by the infectious agent. That is, an individual immunized by the present invention will not be infected or will be infected to a lesser extent than would occur without immunization. By "cleared" we refer to the decrease in the infections titer of a pathogen following its exposure to humoral or cell-mediated immune responses or humoral and cell-mediated immune responses.

In the subject method, DNA encoding a desired antigen, referred to as a "DNA transcriptional unit", is introduced into a host in whom effective immunity is desired. The DNA transcriptional unit consists essentially of a polynucleotide sequence, which includes antigen-encoding DNA and transcriptional promoter elements. Optional sequences include enhancer elements, splicing signals, termination and polyadenylation signals, viral replicons and bacterial plasmid sequences.

DNA encoding the desired antigen can be inserted into various expression vectors to construct the DNA transcriptional unit, using known methods. See

Maniatis et al., Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press (1989).

The DNA transcriptional unit can be inoculated in the presence of adjuvants or other substances that have the capability of promoting DNA uptake or recruiting immune system cells to the site of the inoculation. It should be understood that the DNA transcriptional unit, itself, will be expressed by host cell factors.

The "desired antigen" can be any antigen expressed by an infectious agent or any antigen that has been determined to be capable of eliciting a protective response against an infectious agent. These antigens may or not be structural components of the agent. The encoded antigens can be translation products or polypeptides. The polypeptides can be of various lengths. They can undergo normal host cell modifications such as glycosylation, myristoylation or phosphorylation. In addition, they can be designed to undergo intra-cellular or cell-surface expression. Furthermore, they can be designed to undergo assembly and release from cells.

Potential pathogens that desired antigens can be derived from include any virus, chlamydia, mycoplasma, bacteria, parasite or fungi. Viruses include the orthomyxoviruses, rhinoviruses, picornaviruses, adenoviruses, paramyxoviruses, coronaviruses, rhabdoviruses, togaviruses, flaviviruses, bunyaviruses, rubella virus, reovirus, hepadna viruses and retroviruses including human immunodeficiency virus. Bacteria include mycobacteria, spirochetes,

rickettsias, chlamydia, and mycoplasma. Fungi include yeasts and molds. Parasites include malaria.

An individual can be inoculated through any parenteral route. For example, an individual can be inoculated by intravenous, subcutaneous or intramuscular methods.

Any appropriate physiologically compatible medium is suitable for introducing the DNA transcriptional unit into an individual. Suitable mediums include saline.

Immunization of Chickens Against Influenza Virus

Example 1

Procedure:

A DNA transcriptional unit referred to as pP1/H7 (Fig. 1), encoding a replication competent avian leukosis virus expressing the influenza virus hemagglutinin type 7 (H7) gene was constructed as described in Hunt et al., J. of Virology, 62(8):3014-3019 (1988). DNA unit p188 (Fig. 2) encoding a replication defective derivative of pP1/H7 that expresses H7 but is defective for the avian virus vector polymerase and envelope proteins was constructed by deleting an XbaI fragment from pP1/H7. DNA unit pRCAS (Fig. 3), encoding the avian leukosis virus vector, with no influenza virus insert, was constructed as described in Hughes et al., J. of Virology, 61:3004 (1987). DNA units were diluted in saline at a concentration of 100 μg per 0.2 ml for inoculation.

To test the ability of the inoculated DNA to protect against a lethal influenza virus challenge,

groups of three-week old chicks were inoculated with pP1/H7, p188, or pRCAS DNA. Specific pathogen free chicks that are maintained as an avian-leukosis virus-free flock (SPAFAS, Norwich, CT) were used for inoculations. Each chick received 100 μg of DNA (~$1x10^{13}$ molecules) intravenously (iv), 100 μg intraperitoneally (ip), and 100 μg subcutaneously (sc). Four weeks later chicks were bled and boosted with 300 μg of DNA (100 μg iv, 100 μg ip, and 100 μg sc). At one week post-boost, chicks were bled and challenged by the nares with 100 lethal doses ($1x10^4$ egg infectious doses) of a highly pathogenic type H7 avian influenza virus, A/Chicken/Victoria/1/85 (H7N7) (Ck/Vic/85). The chickens were observed daily for ten days for signs of disease. One and one half weeks after challenge, sera were obtained from surviving birds. These as well as the pre- and post-boost sera were used for analyses for hemagglutination inhibiting antibodies (HI).

The testing of the sera were done in microtiter plates with receptor-destroying enzyme-treated sera as described by Palmer et al., Advanced Laboratory Techniques for Influenza Diagnosis, p. 51-52, Immunology series no. 6, U.S. Department of Health, Education, and Welfare, Washington, D.C. (1975).

Results:

The H7-expressing DNA transcriptional units protected each of the chickens inoculated with pP1/H7 or p188 (Table 1). In contrast the control DNA, pRCAS, failed to protect against lethal challenge. The birds

in the control group started to show signs of disease on the second day post-challenge. By the third day, three of the six control birds had died and all control birds were dead by the fifth day. The birds inoculated with hemagglutinin-expressing DNAs showed no signs of disease. By one and one half weeks post challenge both of these groups had developed high levels of HI antibody.

Example 2

Procedure:

The p188-H7 expressing DNA and control DNA were tested again for the ability to protect against a lethal influenza virus challenge. This experiment included a group that was vaccinated and boosted by all three routes of inoculation (i.e., i.p., i.v. and s.c.), a group that was vaccinated by all three routes but did not receive a boost, small groups that were vaccinated and boosted by only one route of inoculation and a control group treated with the anti-influenza virus drug, amantadine-HCL. This last group was included to allow the comparison of antibody responses to the challenge virus in vaccinated and unvaccinated chickens. The amantadine-treated birds were given 0.01% amantadine in their drinking water beginning 8 hours after challenge and were also injected ip with 1.0 ml of 0.1% amantadine 24 and 48 hours after challenge.

RESULTS:

The results of this experiment confirmed that the replication defective H7-expressing DNA could afford protection against a lethal challenge (Table 2). In contrast to the first experiment, the p188 immunized birds showed transient signs of sickness following the challenge. As in the first experiment, the control DNA did not provide protection. All of the 5 amantadine-treated control birds developed disease. Four of these survived the challenge, providing sera that could be used to compare the time course and specificity of anti-influenza virus responses in immunized and non-immunized chickens (see example 4 below).

Example 3

Procedure:

A third experiment was initiated to increase the numbers of birds in the test groups and to further evaluate the efficacy of different routes of immunization. In this experiment 12 chicks were inoculated with p188 by the iv, ip, and sc routes, 8 chicks were inoculated iv-only and 8 ip-only. For controls, 12 chicks were inoculated with pRCAS and 12 chicks were not inoculated. All immunizations were followed by a boost.

RESULTS:

The results again demonstrated protection by p188 (Table 3). Eight of the 12 p188 immunized birds survived whereas all 12 of the control pRCAS chickens died. The twelve birds in the untreated control group also had no survivors. Six out of the 8 chickens inoculated iv-only with p188 survived whereas none of the 8 chickens inoculated ip-only survived.

Example 4

Procedure:

Sera from p188 inoculated and amantadine treated birds in the second experiment were analyzed for the time course of antibody responses to H7 and to other influenza virus proteins (Table 4). Antibody responses to H7 were quantitated using hemagglutination inhibition as well as virus neutralization and enzyme-linked immunosorbent assays (ELISA) for antibody . Neutralizing antibody was determined in chick embryo fibroblast cultures with 200 $TCID_{50}$ of virus using cytopathology and hemagglutinin for detection of virus replication.

RESULTS:

As in the first experiment (Table 1), these assays revealed very low levels of anti-H7 antibodies in the p188 inoculated and boosted chickens (Table 4). These low levels were above background because birds that had low levels of antibody scored for antibody in each of the three assays (HI, neutralization, and ELISA) and

uniformly survived the challenge. Within one week of challenge, high levels of anti-influenza antibodies had appeared in the immunized group. These levels did not undergo much, if any, increase between one and two weeks post challenge. Most of this response was specific for H7. Sera had high titers of ELISA antibody for the H7 influenza virus (Ck/Vic/85) (titer of $1X10^6$) but only low titers of ELISA antibody for the H5 virus (Ck/Penn/83) (titer of $4.6x10^4$) (Table 4). By contrast, in the amantadine-treated control group, antibodies did not appear until two weeks post-challenge (Table 4). As much of this response was against the H5 virus (ELISA antibody titer of $1x10^6$) as against the H7 virus (ELISA antibody titer of $1x10^6$). These results demonstrate that the p188-inoculation had primed an H7-specific antibody in the protected birds.

Table 1

Protection Against Lethal H7N7 Influenza Virus with DNA Coding for H7 Hemagglutinin

		HI TITERS		
Group	Sick/Dead/Total	Post-vaccine 4 weeks	Post-boost 1 week	Post-Challenge 1.5 weeks
pP1/H7	0/0/6	<.[a]	<.	864 (160-1280)
p188	0/0/6	<[b]	<	427 (160-1280)
pRCAS	6/6/6	<	<	+[c]

[a] (<.) means one of six birds had an HI titer of 10.

[b] (<) means that all birds had titers of less than 10.

[c] (+) means that all birds died.

TABLE 2

Protection Against Lethal H7N7 Influenza Virus with DNA Coding for H7 Hemagglutinin

Group	Route of Inoculation	Boost	Sick/Dead/Total[a]
p188	ip/iv/sc	yes	6/1/6
p188	iv only	yes	1/1/2
p188	ip only	yes	0/0/2
p188	sc only	yes	2/2/2
pRCAS	ip/iv/sc	yes	5/4/5
none	NA[b]	NA	
none Aman.[c]	NA	NA	5/1/5
p188	iv/ip/sc	no	4/4/6
pRCAS	iv/ip/sc	no	6/6/6

[a] Sick birds that survived developed only mild signs of sickness such as ruffled feathers and temporary loss of appetite.

[b] (NA) not applicable.

[c] (Aman.) is an abbreviation for Amantadine.

TABLE 3

Protection Against Lethal H7N7 Influenza Virus with DNA Coding for H7 Hemagglutinin

Group	Route of Inoculation	Boost	Sick/Dead/Total[a]
p188	iv/ip/sc	yes	6/4/12
p188	iv only	yes	2/2/8
p188	ip only	yes	8/8/8
pRCAS	iv/ip/sc	yes	12/12/12
none	NA[b]	NA	12/12/12

[a] Sick birds that survived developed only mild signs of sickness such as ruffled feathers and temporary loss of appetite.

[b] (NA) not applicable

TABLE 4

Antibody Response in H7-Immunized and Amantadine-Treated Birds

Grp.	No.[a]	Bleed	HI	Antibody to Ck/Vic/85 (H7N7) Neutralizing	ELISA ($x10^{-3}$)	Antibody to Ck/Penn/1370/83 (H5N2) ELISA ($x10^{-3}$)
p188	6	1 wk PB[b]	5 (0-10)	2 (0-10)	2 (0-10)	<
	6	2 wk PB	8 (0-20	13 (0-33)	5 (0-10)	<
	5	1 wk PC[c]	112 (80-160)	873 (33-3333)	640 (100-1000)	26 (0-100)
	5	2 wk PC	272 (80-640)	540 (33-1000)	640 (100-1000)	46
None Aman	5	1 wk PB	<[d]		<	<
	5	2 wk PB	<	<	<	<
	4	1 wk PC	<	<	<	
	4	2 wk PC	300 (80-640)	442 (100-1000)	1000 (1000)	1000 (1000)

Antibody titers are given as the median (range).

[a] (No.) Number of chicks in group at time of bleed.

[b] (wk PB) means weeks post boost.

[c] (wk PC) means weeks post challenge.

[d] (<) means all birds had titers of less than 10.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other such equivalents are intended to be encompassed by the following claims.